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COMPARATIVE BIOCHEMICAL AND GENETIC STUDIES OF TESTA DEVELOPMENT IN NORMAL AND HULL-LESS PHENOTYPES OF PUMPKIN (CUCURBITA PEPO L)

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University of New Hampshire PH.D. 1983

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BY

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DISSERTATION

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ABSTRACT

COMPARATIVE BIOCHEMICAL AND GENETIC STUDIES OF TESTA DEVELOPMENT IN NORMAL AND HULL-LESS PHENOTYPES OF PUMPKIN (CUCURBITA PEPO L.)

by

Susan G. Stuart

University of New Hampshire, May, 1983

A detailed investigation was conducted to examine further the biochemical and genetic bases of the hull-less phenotypic condition in testae of seeds of pumpkin (Cucurbita pepo L.). During seed development, testa moisture declined substantially (30%) in both phenotypes, whereas testa dry and fresh weights increased dramatically between 10 to 20 days post-anthesis and then declined steadily until the seeds reached maturity. Structural compositional analysis revealed marked reductions in lignin (78%) and cellulose (50%) contents in hull-less as compared with normal phenotypes; whereas no differences in the non-cellulosic polysaccharides, hemicellulose and pectins were found. About 40% of the testa of normal and hull-less strains was manifested as these two fractions at 20 days post-anthesis.

Between 20 days post-anthesis and maturity, the pectin content of testae was dramatically reduced in both phenotypes (80-90%). A similar reduction was observed for both hemicellulose fractions, although the reduction was greater for mutant strains (80%) than for normal strains (50%).

Analyses of precursor pools and reserve storage material at 20 days post-anthesis did not reveal any significant or consistent

differences in quantities of sugars, free amino acids, total phenolics, lipids and starch between phenotypes. By maturity, precursor pools and starch within the testa had been essentially exhausted in all strains investigated.

Total N values for testae of normal and hull-less phenotypes were not consistently different at 20 days post-anthesis and maturity. About 60% of total testae N compounds was recovered as soluble fractions at 20 days post-anthesis. A substantial amount of structural protein was bound to the hemicellulose'A' fractions and substantially lesser amounts were recovered from cellulose, pectin, and hemicellulose'B' fractions of cell walls in normal and hull-less strains as determined by amino acid analysis. No consistent differences in amino acid composition of the hemicellulose'A'-associated protein were observed between phenotypes. Some differences in amino acid composition of the hemicellulose'B'-associated protein were observed between the normal and hull-less strains, however, discrepancies in amino acid composition between replicates preclude making definitive conclusions about the nature of the differences at this time. The amino acid composition of DOC-soluble protein did not exhibit consistent differences between phenotypes, although some variation in composition was evident within phenotypes.

Results of the inheritance investigation indicate that the hull-less developmental condition in testae of seeds of pumpkin is controlled by a single major gene, and the degree of hull-lessness may be affected by modifying genes (hull-less intensifiers). Variable expression of the hull-less phenotypes indicates some degree of environmental interaction with hull-less genotypes.

INTRODUCTION

Pumpkin seeds (Cucurbita pepo L.) normally develop a well-defined testa or seed coat (Singh and Dathan, 1972) by 10 to 15 days post-anthesis (Stuart, 1981). Tschermak-Seysenegg (1934) reported a "naked-seeded" or hull-less mutant of C. pepo which did not develop a distinctly defined testa. Rather, the testa of the hull-less mutant phenotype exists as a thin chlorophyllous layer of tissue with variable degrees of slight hull formation in the testa margin. Numerous investigations have been conducted to determine the genetic basis for the hull-less condition, although a general concensus has not been reached (Grebenscikov, 1954; Heinisch and Ruthenberg, 1950; Mudra and Neumann, 1952; Schöniger, 1950; 1952; 1955; Weiling and Prym von Becherer, 1950).

Previous comparative biochemical analyses of testae composition of mature seeds of C. pepo revealed marked reductions in lignin (83%), cellulose and hemicellulosic polysaccharides (66%), and significant increases in ethanol-soluble material (58%) and lipids (45%) in hull-less compared with normal strains (Stuart, 1981; Stuart and Loy, in press). Histochemical investigations of developing testae showed no differences in development between normal and hull-less phenotypes until ca. 15 to 20 days post-anthesis (Stuart, 1981). Because of marked phenotypic differences at this stage of development, it seemed appropriate to utilize such testae for further biochemical investigation of hull-less metabolism. Trends in development and utilization of soluble and structural fractions could subsequently be examined in normal and

and hull-less phenotypes. More importantly, any differences obtained could reflect a possible metabolic effect(s) of the genetic lesion(s) responsible for the hull-less developmental condition.

Further investigations of the hull-less mutant trait were undertaken with the following objectives: 1) to compare the magnitude of testa development in normal and hull-less strains during discrete time periods of seed development by determining testa fresh weight, dry weight and percentage moisture; 2) to investigate amounts of carbohydrate, lignin and protein precursor in hull-less and normal strains; 3) to determine the composition of testae at 20 days post-anthesis in normal and hull-less strains; and 4) to conduct an inheritance study using different normal and hull-less strains of Cucurbita pepo to determine the genetic nature of the hull-less condition in testae of pumpkin.

LITERATURE REVIEW

Introduction to Seed Development in Angiosperms

A true seed is derived from a mature, fertilized ovule and the latter usually differentiates into four morphologically distinct components: (i) the embryo which is the fertilization product of ovum and male pollen nucleus unification, containing essentially all the genetic information and requisite stored nutrients (exclusive for cereals) for seed development and differentiation into a mature plant; (ii) the endosperm, a triploid tissue resulting from the fusion of two polar female nuclei and a single generative male nucleus, which functions as a nutritive source during early stages of embryogenesis or as the major reserve material in some seeds; (iii) the perisperm, a nucellar tissue derivative which also serves a nutritive function for the developing embryo; and finally, (iv) the testa or seed coat, a derivative of either or both ovular integuments, which differentiates early in seed development and eventually encases the developing embryo.

Testa Structure and Development in Cucurbita Pepo

The seeds of all Cucurbita species develop from a bitegmic ovule; however, the inner integument disentegrates very early in seed differentiation (Singh, 1953; Singh and Dathan, 1972; Esau, 1977). Hence, the testa or seed coat is derived exclusively from the ovular outer integument which is six to eight cell layers thick. The four to six innermost cell layers divide and differentiate to form the

parenchyma and chlorenchyma tissues of the testa. The epidermis of the outer integument undergoes two successive periclinal divisions, resulting in three layers designated, e, e'', and e' from outside to inside (Singh and Dathan, 1972). These epidermal divisions may commence even in unfertilized ovules (Singh, 1953).

Further development of e' results from a series of periclinal divisions. The cells enlarge tangentially, sclerify and form the main mechanical layer (sclerenchyma) of the testa (Singh, 1953). The cells of e and e'' divide tangentially forming four to six cell layers. The outermost derivative (epidermis) of the differentiating testa, e, experiences radial enlargement, the degree being strain-specific (Stuart, 1981), as well as dependent upon location of the particular cell in the testa, i.e. margin versus center of the seed (Singh and Dathan, 1972). The remaining derivatives of e and e'' form the seed hypodermis.

At maturity, the testa is differentiated into five distinct zones or tissue regions: 1) the epidermis, a single layer of radially elongated cells ca. 100 to 600 μm long; 2) the hypodermis, which is variable in thickness, consists of three to five layers of tightly packed, reticulated and lignified cells; 3) the sclerenchyma, consisting of one layer of slightly branched, markedly lignified cells; 4) the parenchyma, three to four layers of loosely packed, sparsely lignified cells which also exhibit a reticulated appearance; and 5) the chlorenchyma, a multitude (8 to 10 cell layers) of thin-walled, tangentially elongated cells, containing some starch granules and plentiful amounts of protochlorophyll. The innermost layer of the

chlorenchyma tissue represents the inner epidermis of the outer integument.

The testa in the seed margin normally contains a significantly greater number of cell layers compared with the seed surface. This difference is specifically attributable to more hypodermal, sclerenchyma and parenchyma layers (Stuart, 1981).

In dry, mature seeds, the chlorenchyma can be separated from the remaining tissues of the differentiated testa as a thin, translucent green membrane. This tissue together with the inner epidermis closely envelops the embryo and the nucellar and endosperm remnants (Esau, 1977).

The Role of the Testa in Embryogenesis- Is There Any?

Considerable attention has been given to anatomical and morphological investigations of testae, as well as seed development in several genera of angiosperms (Bhatnagar and Johri, 1972), including extensive studies of Cucurbita (Barber, 1909; Singh, 1953; Singh and Dathan, 1972). During the past couple of decades, the biochemical, physiological and molecular bases of embryogenesis, particularly in legume species (e.g. Pisum, Vicia and Glycine), have become the focus of intensive research (reviewed in detail by Dure, 1975). In light of these advances, however, an extreme paucity of literature exists concerning the developmental function and significance of the seed testa.

Following a review of the literature, one might consider the testa a type of 'vestigial' organ, serving only to protect the newly-

formed embryo (Esau, 1977; Khan, 1977) and perhaps aid in maintaining and/or breaking dormancy in some species once the seed has matured (Van Sumere, 1960; Ohkuma et al., 1963; Edwards, 1968; Brown and Van Staden, 1975; Scheibe and Lang, 1975; Evanari and Stein, 1953). This maternally-derived organ, however, is not a simplistic monolayer of cells, but rather it is an intricate construct of specialized tissues. In Cucurbita pepo, five distinct tissues are observed early in embryogenesis, surrounding the embryo sac, attenuated cells of the nucellus, and endosperm (Stuart, 1981). To exemplify evolution of the testa, the primitive ancestors (Cucurbita texana and C. andreana) of domesticated species of Cucurbita, C. pepo and C. maxima, exhibit testae with primitive features such as fewer hypodermal layers, slanting sclerids and a weakly developed aerenchyma (Singh and Dathan, 1972). Why then in Cucurbita species, and generally applying this to any species, would the seed have evolved with such an elaborate testa if a simplified monolayer could have satisfied the need for a protective covering?

Starch, which could eventually be assimilated by the developing embryo, is synthesized and apparently stored in the developing testa of C. pepo (Stuart, 1981; Stuart and Loy, in press). Mature testae of C. pepo do not exhibit any amyloplasts. Hence, some mechanism(s) for hydrolysis of these starch bodies and eventual polysaccharide mobilization must exist, whether the products are incorporated into the growing walls of the testa, or alternatively, are translocated to the nucellus and ultimately utilized by the embryo. Generally, however, the testa appears to have been avoided, although not purposely, in

most investigations of embryogenesis. Raacke (1957) investigated the flow of nitrogenous compounds during pea embryogenesis (Pisum sativum) and essentially ignored the testa. Flinn and Pate (1968) performed elaborate studies of protein and amino acid levels in tissues of the pod and developing seed, without an explanation or even a suggestive role of the testa. These investigators were primarily concerned with nutrient transport during embryogenesis, reporting that there must be a continuous flow from the plant to incipient embryo, since the gain in nitrogen compounds by the embryo greatly exceeded the loss of these compounds by remaining tissues of the developing seed.

Because the developing testa represents a direct transport pathway between the developing embryo and vegetative plant (via the vascular bundles of the testa), logic dictates that some, perhaps many, nutrients could be 'housed' in this organ until needed by either the growing walls of the testa tissues or by the embryo when nucellar and/or endosperm reserves have been depleted.

Practically all developing embryos come "equipped" with a suspensor, and in many species of angiosperms where the cotyledon cells "are the major repository of assimilates" (Gunning and Pate, 1974), specialized suspensor transport cells have been reported (Gunning and Pate, 1974; Graham and Gunning, 1970). The suspensor tissue consists of a collection of specialized cells whose sole function is apparently transport of assimilates between vegetative plant, and developing embryo. Because these cells are located directly beneath the developing embryo or embryo sac, they have been affectionately termed, 'suspersors' and, hence, suspend the embryo. Although cucurbits exhibit

embryo cotyledons serving as storage organs, no such specialized cells have been reported to exist. "Transfer aluerone cells" have been reported in wheat (Rost, 1970) occurring adjacent to the remnant of the dorsal vascular bundle of the ovary. He suggested that these cells aid in absorption of mobilized reserves through the vascular bundle to the developing embryo.

Epidermal transfer cells of the embryo also exist in some species. Gunning and Pate (1974) allude to the presumed source of assimilates for these transfer cells as being located in the integument, and that "rate limitation in transfer is more likely at sites of supply and release from the donor tissues of the sporophytic integuments" (testa derivatives).

Corner (1951) reported that the fruit wall and seed coat of leguminous seed must contribute in some measure photosynthetically to the requirements of the embryo, although he never suggested how and to what extent the testa participated in embryogenesis. Corner also reported that beneath the outer photosynthetic layer in Cucurbitaceous seed, a "woody layer" differentiates which is excised from the embryo and, upon desiccation, contains many useless starch grains. Starch grains were not observed in mature Cucurbita pepo testa (Stuart, 1981).

In their review on the biochemical and physiological nature of transfer cells, Pate and Gunning (1972) concluded that physiologists remain "woefully ignorant" of the function, if any, of the internally located types of transfer cells. They also state that the issue must be settled as to whether these specialized cells do really play a role in solute transport. Similarly, the role of the testa in

supplying nutrients to the developing embryo must be more thoroughly investigated.

The Genetics of Testa Development in Cucurbita Pepo

The genetic basis for the hull-less condition in Cucurbita pepo has been investigated by several researchers, but a generally acceptable concensus on the mode of inheritance has not been reached.

Schöeniger published the first of a series of three papers regarding inheritance of the hull-less testa characteristic in 1950. Based on F_2 and F_3 analyses, she proposed a principal or major gene, "H", and a subsidiary dominant gene, "N" governing lignification in cell walls of testa tissues. Subsequently, she reported results of crosses between Zucchini (normally lignified seed) and styrian oil pumpkin, a hull-less mutant (Schöeniger, 1952). All of the F_1 progeny exhibited various degrees of lignification. To account for these observations, Schöeniger stated that apparently the oil pumpkin, although recessive for both lignification genes, "H" and "N", had modifiers which reduced lignification in plants containing the dominant gene "N". F_3 and backcross progeny confirmed this hypothesis. In her third study, Schöeniger (1956) reported two groups of modifiers in homozygous recessive varieties exhibiting the hull-less phenotype. She postulated one set of modifiers active during the commencement of lignification, and a second set exerting influence once lignification had been initiated. In either instance, both sets of modifiers apparently diluted the effect of the subsidiary dominant gene, "N".

Weiling and Prym von Becherer (1950) reported that three layers

of the mutant testa were present as observed in normal seeds, but that the layers failed to exhibit any degree of lignification. They tentatively reported three genes as being responsible for the failure of mutant cell walls to lignify.

Mudra and Neumann (1952) examined inheritance patterns of several testa characteristics, including testa thickness. Based on progeny segregations, they reported that more than two genes were responsible for conditioning the extent of testa thickening.

Grebenscikov (1954) analyzed available data concerned with inheritance of testa thickness in Cucurbita pepo, and additionally, included some personal observations in arriving at his hypothesis. He concluded that testa thickness in the hull-less mutant was determined by a single recessive gene, but that modifiers may exert a limited influence on its expression.

Whitaker and Davis (1962) have attempted to reconcile all the available data and proposals for inheritance of the hull-less mutant phenotypes in seeds of pumpkin. There are apparently slight differences in testa thickening among different hull-less C. pepo strains (personal observations) and thus, crosses between different C. pepo strains may give dissimilar genetic results (Whitaker, 1962; Loy, personal communication). Having examined all the data, however, these investigators prefer to accept monofactorial recessive inheritance of the hull-less mutant phenotype as postulated by Grebenscikov (1954). Additionally, they point out that different modifiers or groups of modifiers may affect testa thickness during precise developmental stages.

Selected Biochemical Aspects of Cell Wall Development

The plant cell wall is a very complex and dynamic organelle which undergoes incessant biochemical and physiological alterations throughout its programmed differentiation. The composition of the wall is also highly variable, making structural investigation quite difficult to pursue, and ultimately, results difficult to interpret.

The wall generally consists of polysaccharide and protein polymers in young cells, or cells which form only primary walls. Lignin and additional polysaccharides are integrated into the primary structure as the cell differentiates further (begins to form secondary walls). "Lignin and additional polysaccharides" may appear to be a concise and easily interpreted phrase. However, the structure of lignin as it exists *in vivo* has not been reported and the quality and quantity of polysaccharides that could be utilized as wall precursors are infinite.

Composition of the Primary Cell Wall

The continous matrix of the primary cell wall consists of two groups of polysaccharides, the hemicelluloses and the pectic polymers, also known as the non-cellulosic polysaccharides. The microfibril mesh which integrates within this continuous matrix consists exclusively of cellulose molecules.

The structural overlap of polymer building blocks among the polysaccharides of the continuous matrix has resulted in arbitrary definitions of each of these polysaccharides. These definitions are based on the monosaccharide precursors involved in complex polysaccharide

formation (Albersheim, 1976; Keegstra et al., 1973). The primary wall hemicelluloses generally consist almost solely of xyloglucan (Talmadge et al., 1973), although a second structurally distinct hemicellulose, glucuronarabinoxylan had been isolated from suspension-cultured sycamore cells (Darvill et al., 1980).

The complexity of the pectic polysaccharides transgresses discussion in this review. However, all of these polymers consist essentially of polymers of galactan, arabans, and methyl esters of polygalacturonic acids (Albersheim, 1976). These cell wall components are highly soluble in boiling water, as well as in very dilute solutions of ammonium oxalate, the latter acting to chelate divalent cations which bind the negatively charged uronic acid molecules when ionized.

Only five sugars (monosaccharides) and five sugar derivatives have been implicated as precursors in primary cell wall biogenesis (Salisbury and Ross, 1978). The sugars are D-glucose, D-galactose and D-mannose (hexoses) and D-xylose and D-arabinose (pentoses). The sugar derivatives are: L-rhamnose, L-fucose, the uronic acids of glucose and galactose, and the methyl ester of galacturonic acid. Myo-inositol and sucrose participate indirectly in cell wall formation as intermediates in the pathways of polysaccharide synthesis (Lamport, 1970). One crucial intermediate is the sugar nucleotide, UDP-D-glucose (Karr, 1976).

In addition to these polysaccharide components of the cell wall, a specific structural protein also exists as an integral part of the

network. This protein, frequently referred to as extensin has been characterized by a high degree of glycosylation, in addition to containing substantial amounts of the hydroxy-amino acids, namely, 4-hydroxyproline, threonine, and serine (Talmadge et al., 1973; Lamport, 1970).

Based on Albersheim's work (1976) with suspension-cultured sycamore cells which only develop primary cell walls, the proportions of different cell wall components have been reported as follows: pectic polysaccharides- 36%; cellulose- 23%; xyloglucan (hemicellulose)- 21%; protein- 10%; and a tetra-arabinoside- 10%. Because the latter molecules are present in amounts stoichiometrically equivalent to protein, Akiyama and Kato (1977) have proposed that the arabinoside participates in glucosidic bond formation between arabinose and hydroxyproline.

The Structural Significance of Protein

Much of the previous work with cell wall analysis has focused on the inter-relationships between polysaccharides (Albersheim and Jones, 1972; Darvill et al., 1980; McNeill et al., 1981). Protein also appears to be intricately involved in the biogenesis of cell walls (Preston, 1974), although uncertainty remains as to which monosaccharide residues it may or does bind. As stated previously, the protein usually contains hydroxyproline, often in a sequence of four residues along the polypeptide chain, with monosaccharide residues subsequently located on either side of this tetra-proline peptide (Franz and Haas, 1980; Preston, 1974). Lamport (1970, 1973) suggested that the proline residues carry arabinose residues of variable length, while serine is

associated with galactose moieties, also of undescribed length. Glycoproteins which are alkali-extracted from beans (Phaseolus vulgaris) have been shown to contain arabinose, galactose and glucose attached to serine residues (Brown and Kimmins, 1973).

The supposition that protein is indeed covalently bound to structural polysaccharides has been substantiated by observations that in some plant tissues glycoproteins can be released by alkali treatment (Knee, 1975; Selevendran, 1975; Selevendran et al., 1976) as well as by delignification treatments (Dreher, 1976). Based on these observations then, some proteins are integrally entangled in the polysaccharide components of the cell wall. It is generally accepted that structural protein binds to some hemicellulose fraction of the wall, although the specific fraction is species- and even tissue-specific (Talmadge et al., 1973; Monro et al., 1974). Dreher (1976) reported that a substantial portion of structural protein is bound to hemicellulose fractions of some Cucurbita species. Based on results with sycamore tissue, Keegstra et al. (1973) have questioned whether the protein-polysaccharide linkage is covalent. Covalent linkage of structural protein to cellulose microfibrils has been reported to exist in lupine hypocotyls (Monro et al., 1976).

Lignin- Its Involvement In Cell Wall Development

Lignin is a very high molecular weight polymer consisting of the alcoholic phenylpropane derivatives, coumarol, sinapol and coniferol (Freudenberg and Neish, 1968). The hydroxyl moiety of these alcoholic precursors is capable of being methylated and thus, the methoxyl

content and relative proportions of alcohol precursors are the distinguishing features of the lignin associated with a plant species. Lignin is synthesized and deposited within the developing secondary wall in close association with the polysaccharide and protein(?) matrix, whereby phenolic hydroxyls have the ability to hydrogen bond or covalently link to polysaccharide or perhaps protein (Harborne, 1979).

Lignin penetrates the primary wall proceeding from the outside inward at very early stages of secondary wall development (Esau, 1977). In developing pumpkin seeds, lignification is observed as early as fifteen days post-anthesis in certain testa tissues (Stuart, 1981). Partially because of its hydrophobic nature, it replaces water in the cell wall as biogenesis proceeds, encrusting the microfibril-polysaccharide matrix, as well as between the cellulose microfibril-continuous matrix interface (Northcote, 1972).

Biochemical Basis For the Hull-less Condition In Seeds of C. Pepo

Previous testa analyses of mature normal and hull-less mutant strains of Cucurbita pepo indicated a marked reduction in lignin (83%) as well as dramatic reductions in cellulose and hemicelluloses (66%) in hull-less strains as compared to hulled strains (Stuart, 1981). Both cellulose and hemicelluloses are integral components of the primary and secondary cell walls, whereas lignin is generally associated with secondary wall formation. Preston (1974) postulated the importance of a polysaccharide framework as a prerequisite for lignin biosynthesis. Thus, it has been proposed that lignin deposition in the hull-less C. pepo mutant is decreased indirectly as a result of

insufficient polysaccharide formation in testa tissue cell walls. This hypothesis differs from that of previous investigators (Schöeniger, 1950; 1952, 1955; Grebenscikov, 1954; Mudra and Neumann, 1952; Weiling and Prym von Becherer, 1950) who proposed that a deficiency in lignification was the primary effect of the hull-less genetic lesion(s).

Testa tissues of mutant strains also contain significatly reduced amounts of protein (54%) compared to normal strains (Stuart, 1981). As examined previously, protein can bind to hemicellulose components of the cell wall as well as to polysaccharide fractions (Dreher, 1976) and perhaps even to lignin (Harborne, 1979). Since these fractions are considerably reduced in hull-less testae, the observed reduction in protein is not suprising. Whether this reduction would be a cause for the mutant condition or an effect of decreased quantities of hemicellulose remains to be determined. In any case, because a substantial portion of the testa is protein and also because the protein fraction is apparently bound to hemicelluloses and perhaps other wall fractions, it logically follows that protein should be intricately involved with cell wall differentiation or biogenesis of testae of Cucurbita pepo.

MATERIALS AND METHODS

Plant Material and Growing Conditions

For the inheritance study, seeds of two normal strains, cvs. Jack O'Lantern (Northrup King and Co., Minneapolis MN) and Small Sugar (Stokes Seed Co., Buffalo, NY), three hull-less cultivars, Tricky Jack (E.M. Meader, Rochester, NH), 293 A (Stokes Seed Co., Buffalo, NY) and Triple Treat (Burpee Seed Co., Warminster, PA) and three inbred hull-less or partially hull-less lines, NH 61-22-19, NH 61-22-19-12 and NH 10-2-28 (J.B. Loy, Department of Plant Science, UNH, Durham) of Cucurbita pepo L. were utilized. F_2 seed was collected from fruits of F_1 plants grown in the UNH greenhouses. F_3 seed was collected from fruits of F_2 plants grown at the Horticulture Farm, Durham, NH during the summer of 1982.

For biochemical and developmental studies, the cultivars Small Sugar and Jack O'Lantern and hull-less mutant cvs. Tricky Jack and 293 A were used. Seed collection was at five day intervals beginning at ten days post-anthesis and terminating at thirty days post-anthesis. Mature seed (55 days post-anthesis) was also collected. Seeds from 2 to 3 fruit of each variety were removed, cleansed in distilled water, and stored in polyethylene bags at -10 C until analyzed. All pollinations were done by hand. Male and female flowers were closed with 'twist-ems' on the day prior to pollination, and female flowers were re-tied following pollination.

Inheritance Study

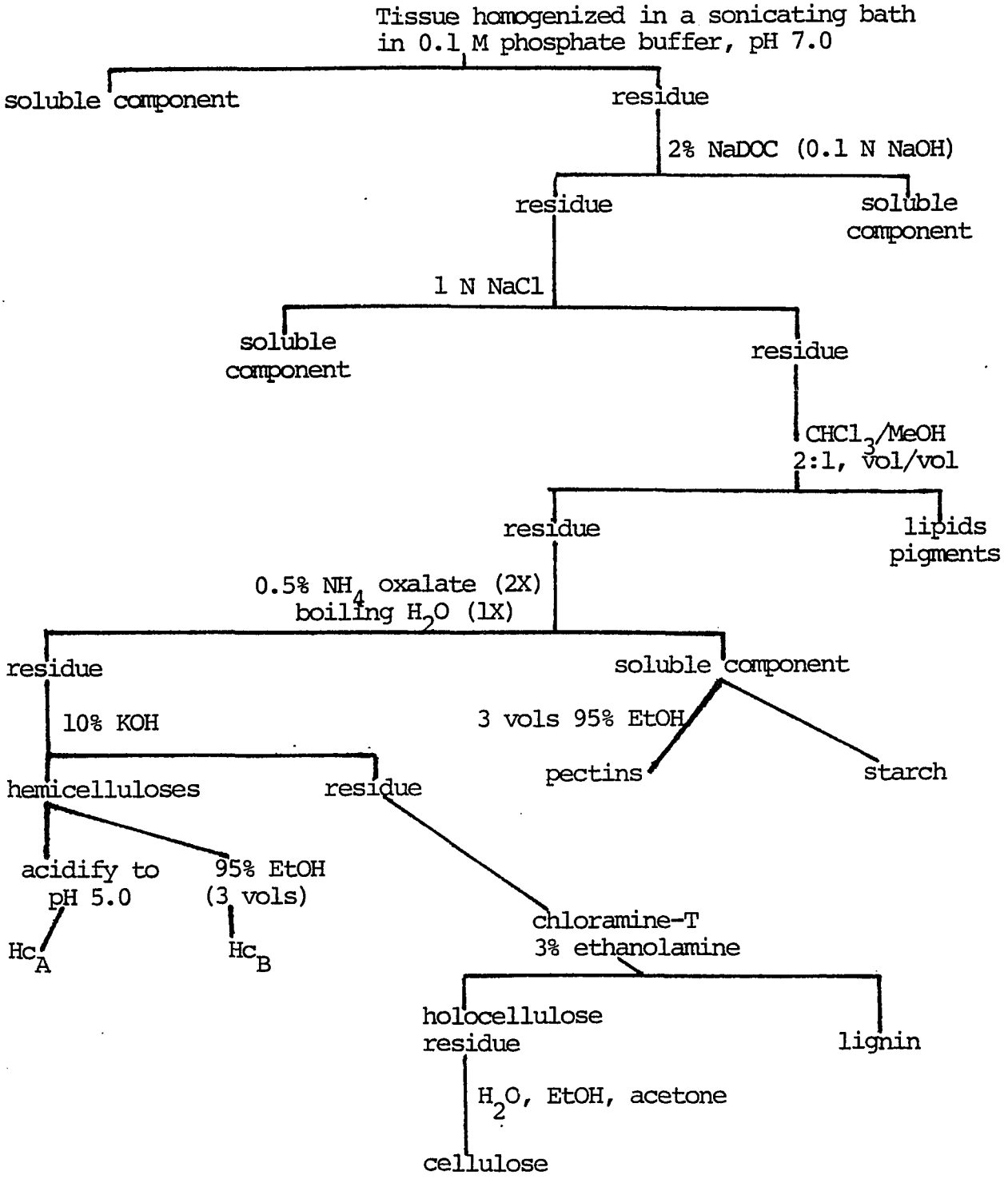
Three F_2 populations and two testcross populations were used for the inheritance study. Seed samples consisting of ca. 10 seed/fruit were removed from each selfed F_2 and test crossed plant, dried at 30 ± 2 C with ventilation and scored for the hull-less or partially hull-less phenotypes. Chi square analyses were subsequently performed on segregating F_2 and test cross populations, using one, two and three gene models for determining expected ratios.

Cell Wall Composition

Cell wall extraction procedures which were used previously (Stuart, 1981) were modified to remove buffer-soluble, salt-soluble and membrane-bound proteins and free nitrogenous compounds, e.g. amides, unbound amino acids, prior to major wall fraction analyses. A flow chart of the extraction procedure is diagrammed in fig. 1.

Developing testae were removed from stored, frozen (- 10 C) seed using gloves to prevent contamination. Samples consisting of ca. 100 to 150 testae were immediately dried in an oven at 65 ± 2 C for 24 h, ground in a Wiley mill (60 mesh) and desiccated over $CaSO_4$. Tissue samples of both normal and hull-less cultivars consisting of ca. 1.0 to 1.5 g of tissue were first extracted in 15 ml of 0.1 M phosphate buffer, pH 7.0. Samples were sonicated in a Branson model B-12 sonicating bath for ca. 30 sec, allowed to stand for 20 min and centrifuged at 12,000 x g for 20 min. Supernatant fluids containing buffer-soluble compounds were carefully pipetted off, volumes were measured and supernatants were frozen at - 10 C for subsequent

Fig. 1. Flow chart for extraction of bound and soluble protein and cell wall components from testa tissue of Cucurbita pepo.



protein, free nitrogen and sugar analyses. Each residue was then extracted in 15 ml of sodium deoxycholate (DOC) in 0.1 N NaOH, the mixtures sonicated for 30 sec, and allowed to stand for 20 min. Mixtures were subsequently centrifuged at 20,000 x g for 30 min. Supernatants were pipetted off and frozen for subsequent soluble protein, free N and sugar analyses. A final extraction was performed on the remaining residues with 1.0 M NaCl. Fifteen ml of the salt solution were added to each residue, the mixtures were sonicated and allowed to stand for 20 min and subsequently centrifuged at 12,000 x g. Supernatant fluids were pipetted off and frozen for free N, soluble protein and sugar quantitation. Following this three-step sequential extraction, residues were dried in an oven at 70 C for 60 h and desiccated over $CaSO_4$ for 24 h in preparation for extraction of lipids and pigments which were not solubilized by sodium deoxycholate.

Lipids and pigments were solubilized as described previously (Stuart, 1981) in chloroform : methanol (2:1, vol/vol). To separate starch from pectic substances, their similar solubility properties were exploited. Dried and weighed lipid-free tissue samples were first extracted in 15 ml of 0.5% aqueous ammonium oxalate at 80 C for 30 min. Mixtures were centrifuged at 12,000 x g for 20 min, supernatant fluids pipetted off, and the extraction and centrifugation were repeated. Each residue was subsequently boiled in 15 ml of hot double-distilled deionized water for 1 h to solubilize remaining starch and perhaps some pectins. Mixtures were centrifuged at 12,000 x g for 20 min and supernatants combined with the two previous ammonium

oxalate supernatant fluids. Ninety-five % ethanol was added to the combined extracts until the concentration of alcohol was ca. 50%. Mixtures were allowed to stand at room temperature for 2 h and resultant precipitates were pelleted by centrifugation at 12,000 x g for 20 min. Most of the supernatant was decanted in each instance and the remaining mixtures were filtered through tarred #2 Whatman filter paper using a Büchner funnel. Supernatant fluids and precipitates were tested for the presence of starch using I_2KI_3 (Jensen, 1962). Filter paper containing pectic substances was dried for 12 h at 70 C and pectin dry weights were obtained. Starch was quantified by first subtracting the pectin weights from the tissue dry weights prior to extraction, and then the remainder of the difference in dry weight before and after ammonium oxalate and boiling water extractions was attributable to starch.

Hemicelluloses were extracted in 20 ml of 10% KOH under nitrogen as described previously (Stuart, 1981). Mixtures were shaken for 24 h, and filtered through a coarse porosity sintered glass funnel. Filtrates were removed and saved. The residue was washed three times (each) with 95% ethanol and acetone. Filtrates were cooled in an ice bath to ca. 20C and the hemicellulose 'A' fraction (Hc_A) precipitated upon acidification to pH 5.0 with the addition of 50% acetic acid. The precipitate was collected by centrifugation at 12,000 x g for 15 min. Supernatants were diluted with three volumes of 95% ethanol to precipitate the hemicellulose 'B' fraction (Hc_B). This fraction was collected by centrifugation at 12,000 x g for 15 min. Both fractions were washed three times each with ethanol and acetone and

and dried for 12 h at 60 C on pre-weighed and dried filter paper. Amounts of each fraction were gravimetrically determined.

Lignin was solubilized from dried residues as described previously (Stuart, 1981) using chloramine-T and 3% ethanolamine. After cooling in an ice bath, reaction mixtures were centrifuged at 12,000 x g for 10 min and the residue (holocellulose) was washed repeatedly with ethanol and acetone and dried for 12 h at 70 C.

The extraction series was performed in duplicate for each strain.

Free (Non-protein) N, Soluble Protein N and Cell Wall (Structural) Protein Analyses

Frozen phosphate buffer, sodium deoxycholate and sodium chloride extracts were analyzed for free nitrogen compounds and soluble protein. Non-protein nitrogen was analyzed utilizing the ninhydrin procedure (Spies, 1957) and results expressed as % threonine N equivalents/ testa. Soluble protein was quantitated by the Bradford procedure (1976) using a bovine serum albumin standard.

The analysis of soluble protein in Na-DOC extracts could not be performed on the original extracts because Na-DOC soluble material was precipitated out of solution by the dilute phosphoric acid in the Bradford reagent. Mixtures were dried and material resuspended in 80% ethanol. Following sufficient vortexing, 100 µl aliquots were used for Bradford protein determination. (See appendix for the complete procedure utilized).

Total N was estimated by Kjeldahl nitrogen determinations. Amino acid analyses of deoxycholate extracts and structural protein contained in the hemicellulose B fraction of testa polysaccharides

were performed by the UNH Instrumentation Center using a Beckman model 118CL amino acid analyzer interfaced to a Varian model CDS-111C integrator. Samples were hydrolyzed in vacuo in 1.0 ml of constant boiling 5.7 M HCl for 24 h at 113 C. A drop of 0.5 M hydrazine was added to each sample prior to analysis to prevent tyrosine degradation. Following hydrolysis, samples were vacuum desiccated over NaOH pellets and concentrated H_2SO_4. Dried samples were resuspended in 1.0 ml of 0.2 N sodium citrate pH 2.2, 0.5% thiodiglycol and 0.1% phenol (Beckman dilution buffer). Norleucine was used as an internal standard and aliquots of samples were injected and chromatographed by single column methodology.

Carbon and nitrogen determinations on deoxycholate extracts were also performed by the Instrumentation Center on dried and desiccated samples consisting of ca. 2.0 to 2.5 mg. Analyses were performed using an atomic absorption spectrometer.

Precursor Pool Analyses

Dried (60C for 24 h) and weighed testa fractions representing 20 days post-anthesis and mature samples were extracted repeatedly with sonication in methanol: chloroform: water (MCW) as described by Haissig and Dickson (1979) (see appendix). Analyses for reducing sugars (Cronin and Smith, 1976), free amino acids (Spies, 1957) and total phenolics (Beck, 1982) were subsequently conducted. The concentration of reducing sugars was expressed as glucose equivalents per testa. Concentrations of free amino acids were expressed as threonine equivalents as described previously, and those of phenolics as phenol equivalents/testa.

RESULTS

Testa Fresh and Dry Weights and Percent Moisture

Testa moisutre gradually decreased during development of both normal and hull-less strains of C. pepo (Fig. 2). Testa moisture was significantly different at the 5% level between normal and hull-less strains at 30 days post-anthesis and maturity as determined by Duncan's Multiple Range tests.

Testa fresh weights increased markedly between 10 and 20 days post-anthesis, peaking at 20 days post-anthesis in all four cultivars investigated (Fig. 3). Between 20 days post-anthesis and maturity, testa fresh weights decreased dramatically in both normal and hull-less mutant strains. The testa fresh weight of cv. Jack O'Lantern was consistently and markedly greater than that of cv. Small Sugar (both normal strains) and 'Tricky Jack' and '293 A' (mutant strains) at 20 and 30 days post-anthesis (Fig. 3). Fresh weights at maturity were lowest in hull-less strains.

Testa dry weights (Fig. 4) exhibited similar developmental patterns to fresh weights. Dry weights increased ca. 2.5-fold between 10 and 20 days post-anthesis, peaked between 20 and 30 days post-anthesis, then declined until maturity in both phenotypes. A significant difference in dry weight between the two normal cultivars was consistently obtained throughout development. At maturity, dry weight of hull-less strains was about 40% that of the average of the two normal strains.

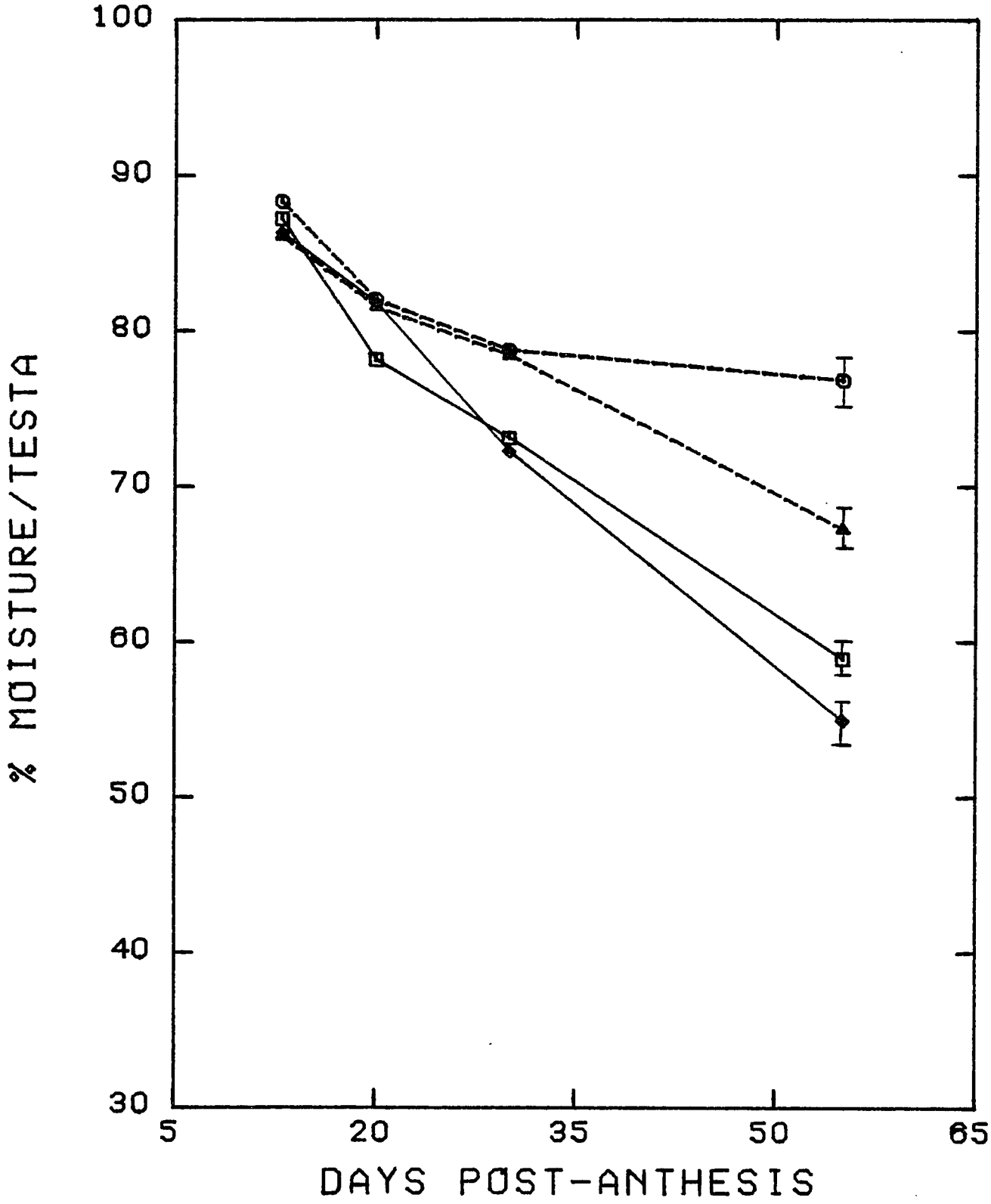


Figure 2. Testa moisture (% moisture/testa) of normal ('Small Sugar'- □—□ and 'Jack O'Lantern'- ◇—◇) and hull-less mutant ('Tricky Jack'- ⬡···⬡ and '293 A'- ◬···◬) strains of Cucurbita pepo. Each point ± SE represents the mean of 3 replicates, 5 testae/rep.

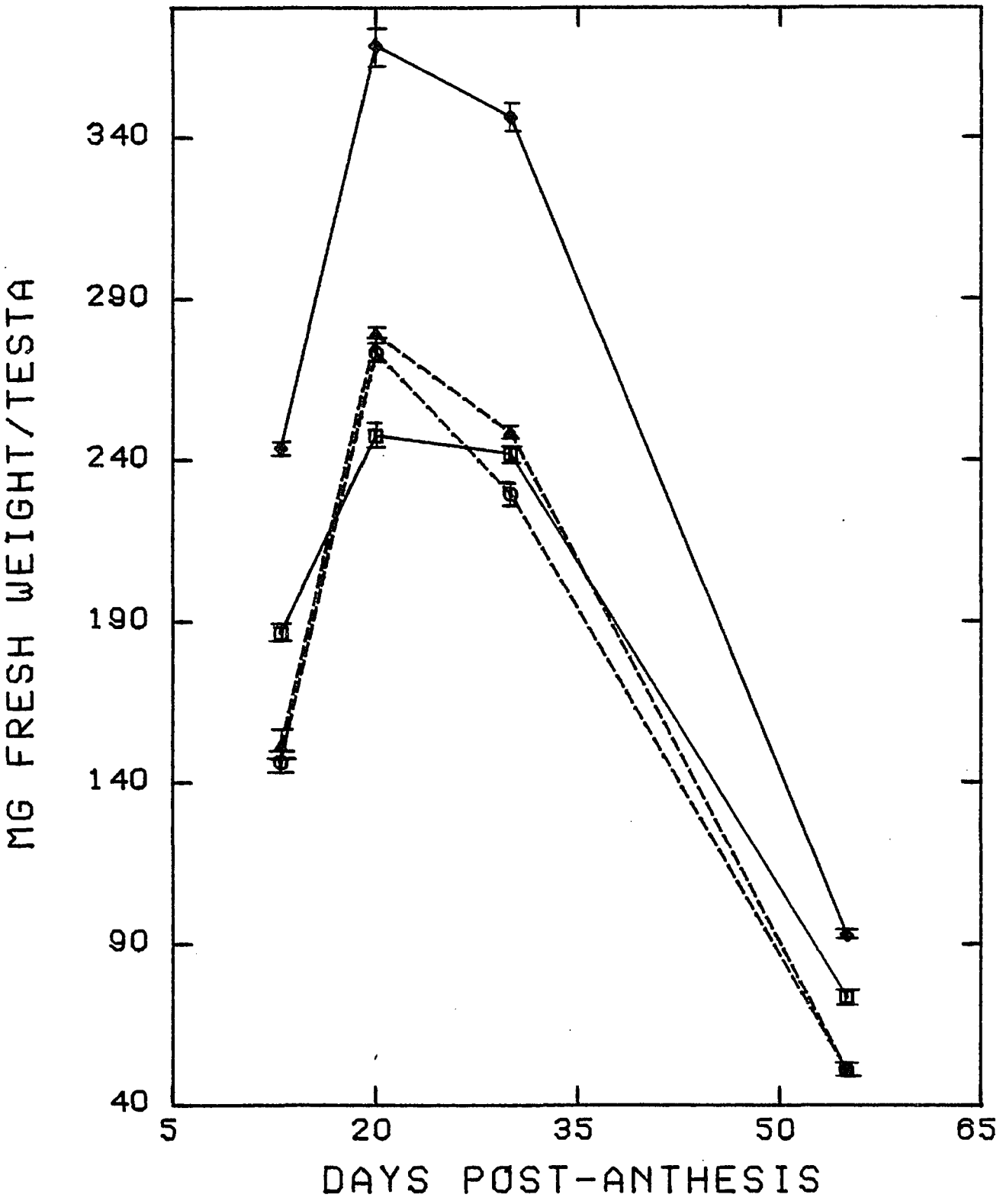


Figure 3. Testa fresh weight (mg fresh weight/testa) in normal ('Small Sugar'-□-□ and 'Jack O'Lantern'◇—◇) and hull-less mutant ('Tricky Jack'-○···○ and '293 A'-△···△) strains of C. pepo. Each point ± SE represents the mean of 3 replicates, 5 testae/rep.

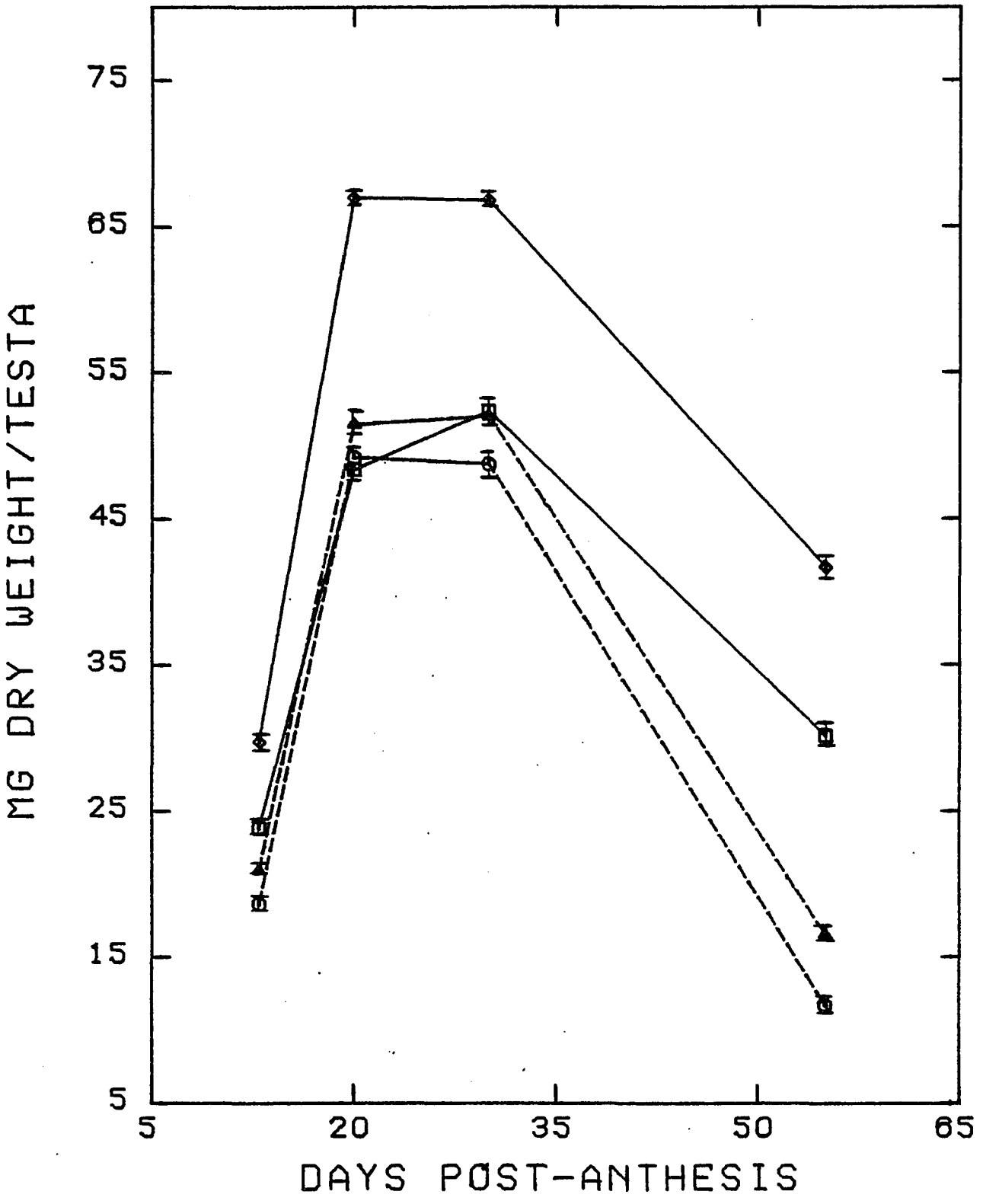


Figure 4. Testa dry weight (mg dry weight/testa) in normal ('Small Sugar'- ▫-▫ and 'Jack O'Lantern'- ◇-◇) and hull-less mutant ('Tricky Jack'○-○ and '293 A'- △··△) strains of C. pepo. Each point ± SE represents the mean of 3 replicates, 5 testae/rep.

Although there was seasonal and yearly variability in testa dry weights and hence, expression of the hull-less phenotypes, relative differences among phenotypes were similar (Table 1). These dry weight differences were primarily attributable to variation in seed size.

Testa Composition

At 20 days post-anthesis, a marked reduction in amounts of lignin(82%) and cellulose (50%) was evident between normal and hull-less mutant phenotypes (Table 2). All remaining structural testae components were quantitatively similar between phenotypes. Matrix polysaccharides contained roughly equal amounts of pectic polysaccharides and hemicelluloses (16-20% for each fraction). The weights of testae hemicellulose fractions and perhaps other fractions included some structural protein which was associated with these wall fractions.

Between 20 days post-anthesis and maturity, a dramatic reduction in pectins (80-90%) was observed for both normal and hull-less phenotypes (compare Tables 2,3). Distribution of testae components in mature seeds was extrapolated from fractionation data of testae obtained using 1981 seed lots. In addition to the reduction in pectic polysaccharide content between 20 days post-anthesis and maturity, a similar reduction in hemicellulose fractions occurred, although the reduction was greater in hull-less (80%) compared with normal (50%) strains (compare Tables 2,3). At maturity, amounts of lignin and cellulose were dramatically reduced (83% and 66%, respectively) in hull-less compared with normal strains (Table 3). The magnitudes of

Table 1. Yearly variability in testa dry weights (mg dry weight/testa) of normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of Cucurbita pepo [a,b]

Development stage	Year	Normal Strains		Mutant Strains	
		'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
20 days post-anthesis	1981	42.3 ± 0.8 B	47.6 ± 3.5 A	35.9 ± 0.5 C	39.1 ± 2.2 B,C
	1982	48.4 ± 1.1 B	67.0 ± 1.0 A	49.2 ± 3.9 B	51.5 ± 0.9 B
55 days post-anthesis	1981	21.2 ± 0.9A	21.5 ± 1.0A	9.5 ± 0.4B	8.1 ± 0.3B
	1982	30.2 ± 0.3B	41.6 ± 2.0A	11.7 ± 0.9D	16.5 ± 1.7C

[a] Means, ± SE of 3 replicates (5 seeds/replication).

[b] Values within rows followed by the same letter are not significantly different according to Duncan's multiple range tests at P = 0.05.

Table 2. Structural components of testae of developing 20 day post-anthesis seeds of normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '203 A') strains of C. pepo (milligrams/testa).

	Normal		Mutant	
Component	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
Pectins	8.2 ± 0.9[a]	11.1 ± 1.5	8.7 ± 0.2	8.2 ± 1.2
Hemicellulose[b]				
- Hc_A	2.1 ± 0.6	3.2 ± 0.1	1.9 ± 0.6	2.8 ± 0.0
- Hc_B	5.5 ± 0.8	9.5 ± 0.4	8.5 ± 0.7	7.3 ± 0.7
Cellulose	5.7 ± 0.2	11.6 ± 0.7	3.8 ± 1.3	3.6 ± 0.2
Lignin	2.5 ± 0.7	3.9 ± 1.2	0.5 ± 0.2	0.7 ± 0.2

[a]Values, ± SD represent the means of two replications.

[b]Weights for each fraction include the weight of bound protein.

Table 3. Structural testa composition of mature seeds of normal ('Small Sugar' and Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of C. pepo (mg/testa).[a]

	Normal		Mutant	
Component	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
Pectins	0.76	0.87	0.54	0.63
Hemicellulose[b]				
$-Hc_A$	1.30	2.12	0.37	0.79
$-Hc_B$	2.78	3.66	0.82	1.14
Cellulose	7.64	11.63	2.49	3.35
Lignin	7.94	9.73	1.17	1.66

[a] Testa fractionation of 1981 seed lots (Stuart, 1981) was used to extrapolate relative proportions of testae components at maturity in 1982 seeds.

[b] Includes the weight of some structural protein associated with these fractions.

these reductions were similar to those at 20 days post-anthesis between phenotypes (Table 2).

Precursor and Reserve Pool Analyses

The methanol/chloroform/water extracts (MCW) obtained from testae of developing 20 day post-anthesis and mature seed of normal and hull-less strains were analyzed for free amino acids, reducing sugars and total phenolics. Starch and lipid contents were also determined but not as part of the soluble MCW fraction. Precursor pool analyses of MCW extracts revealed no significant differences between phenotypes for any precursor (Table 4). Starch and lipids were abundant in testae of both phenotypes, but did not differ significantly between hull-less and normal strains. About 15-20% of the testa dry weight was represented as starch at 20 days post-anthesis, whereas 10-12% was extractable as lipids in both phenotypes.

At maturity, precursor pools were almost completely exhausted in normal and hull-less phenotypes, as were additional reserve materials, lipids and starch (Table 5). Normal strains at maturity contained substantially more starch in testa tissues than mutant strains. Additionally, no accumulation of any precursor was evident at maturity in hull-less mutant testae compared with those of normal strains (Table 5).

Total Nitrogen Analyses

Total nitrogen values for testae of normal and hull-less mutant phenotypes were similar at 20 days post-anthesis. Although the % N of testae was similar at maturity in normal and hull-less mutant strains, actual amounts were reduced in mutant compared with normal strains.

Table 4. Precursor pool and reserve compound analyses of testae at 20 days post-anthesis in normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of Cucurbita pepo.

	Normal Strain		Mutant Strain	
Fraction	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
Precursor compound				
free amino acids (mg/organ)	3.07 ± 0.13[a,b] A	3.74 ± 0.26B	3.05 ± 0.42AB	3.81 ± 0.08B
reducing sugars (mg/organ)	5.44 ± 0.12A	4.97 ± 0.61AB	4.81 ± 0.18AB	4.26 ± 0.36B
phenolics (mg/organ)	0.21 ± 0.01	0.27 ± 0.01AB	0.22 ± 0.01AB	0.33 ± 0.02B
Reserve compound				
starch (mg/organ)	9.15 ± 0.91A	9.95 ± 0.78A	13.05 ± 0.35A	8.60 ± 0.28A
lipids/pigments (mg/organ)	5.80 ± 0.40A	7.50 ± 1.30A	5.70 ± 0.30A	4.52 ± 0.20A

[a] Values ± SE represent the mean of two samples/strain, three replications within each sample.

[b] Values within rows followed the the same letter are not significantly different according to Duncan's multiple range tests at P = 0.05.

Table 5. Precursor pool and reserve compounds at maturity in normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of Cucurbita pepo.

Fraction	Normal Strain		Mutant Strain	
	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
Precursor compound				
free amino acids (µg/organ)	166 ± 4	178 ± 11	183 ± 27	111 ± 5
reducing sugars (µg/organ)	231 ± 4	245 ± 12	370 ± 36	124 ± 10
phenolics (µg/organ)	29 ± 9	22 ± 1	30 ± 1	22 ± 2
Reserve compound				
starch (µg/organ)	841 ± 170	1100 ± 160	384 ± 37	61 ± 11
lipids/pigments (µg/organ)	459 ± 50	1402 ± 40	1108 ± 20	1327 ± 30

[a] Values ± SE represent the means of 2 samples/strain, 3 replications within each sample.

At 20 days post-anthesis, soluble protein and free amino-containing compounds accounted for roughly 30-40% of the total nitrogen in both phenotypes. Non-protein nitrogen compounds represented between 1-2% of the total nitrogen at maturity, while DOC-soluble protein accounted for roughly 10-15% of the total nitrogen in testae.

Analyses for soluble protein (Table 6) revealed no consistent differences between normal and hull-less mutant phenotypes at 20 days post-anthesis. Essentially all soluble protein was recovered in the sodium deoxycholate fractions, regardless of phenotype.

Amino Acid Composition of Testae Protein at Twenty Days Post-Anthesis

Structural Protein

The protein bound to the hemicellulose 'A' fraction represented roughly 40-60% of the total hemicellulose 'A' fraction weight in both phenotypes; whereas a markedly less amount of protein (5-6%) was bound to the hemicellulose'B' fraction of cell walls (estimated from nmoles of amino acids recovered; appendix). No consistent differences were observed in amino acid composition of hemicellulose'A'-associated protein between normal and hull-less phenotypes (Table 7). However, some differences in amino acid composition of structural protein isolated with xylan (HcB) wall fractions were evident between normal and hull-less phenotypes (appendix). Discrepancies in amino acid composition between replicates were evident, however, and further replication is required to resolve these apparent differences between normal and hull-less phenoyptes.

Hydroxyproline which has been implicated as a major component of

Table 6. Soluble protein in 0.1 M phosphate buffer, pH 7.0, 2% sodium deoxycholate (DOC) in 0.1 N NaOH, and 1.0 M sodium chloride extracts, and structural protein estimates of testae of normal ('Small Sguar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of Cucurbita pepo at 20 days post-anthesis.

	Normal		Mutant	
Protein	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
phosphate buffer (µg/organ)	35 ± 13 [a]	35 ± 14	20 ± 9	25 ± 7
sodium DOC (µg/organ)	2110 ± 141	2170 ± 170	2152 ± 155	2105 ± 70
sodium chloride (µg/organ)	116 ± 56	48 ± 13	31 ± 14	51 ± 12
total soluble (µg/organ)	2261	2253	2203	2181
total structural (µg/organ)	2305	7840	4848	3640

[a] Values ± SE represent the means of 2 samples/strain (3 replicates within each sample).

[b] Estimates are based on total Kjeldahl nitrogen determinations X 6.25 minus total soluble protein as determined above.

Table 7. Amino acid composition of hemicellulose'A'-bound protein of testae of normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of Cucurbita pepo.[a,e]

Amino Acid	Normal		Mutant	
	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
aspartic acid[b]	9.8	9.7	9.8	9.9
threonine[c]	5.1	3.9	4.0	4.1
serine[c]	4.3	4.5	4.5	4.5
glutamic acid[b]	10.2	10.3	10.3	10.4
proline[d]	5.1	3.8	5.2	4.6
glycine[c]	6.8	7.3	6.6	7.4
alanine[d]	9.1	11.3	8.5	8.9
½ cystine[c]	0.0	tr	0.0	0.0
valine[d]	8.6	9.4	8.4	8.8
methionine[d]	2.0	1.7	1.8	1.8
isoleucine[d]	7.2	6.6	6.7	6.9
leucine[d]	12.6	11.0	11.4	11.6
tyrosine[c]	3.1	3.3	5.1	3.2
phenylalanine[d]	5.7	5.0	6.3	5.4
histidine[b]	1.8	1.6	1.7	1.8
lysine[b]	4.6	4.6	4.8	5.0
arginine[b]	4.2	4.0	3.5	3.8

[a] Data are expressed as % composition of protein.

[b] Represents a polar, charged amino acid.

[c] Represents a polar, uncharged amino acid.

[d] Represents a non-polar amino acid.

[e] Analysis did notinclude hydroxyproline or tryptophan.

cell wall protein could not be detected with the standard analysis procedure. Proline content of the structural protein(s), however, was similar for both phenotypes (3-4%).

Soluble Protein

The amino acid content was determined for sodium deoxycholate-soluble 'protein' hydrolyzates which had been precipitated out of solution using a very dilute aqueous solution of phosphoric acid. The proportion of N of this precipitate was suprisingly low (1.3-2.0%).

Investigation of the amino acid composition did not reveal any consistent differences between normal and hull-less mutant phenotypes (Table 8). The polar to non-polar amino acid ratio was similar to that observed for structural protein (6.5 : 3.5, Table 8), but several differences among individual amino acids of the structural versus soluble protein(s) were evident. (E.g. the aspartic acid content was significantly reduced in soluble compared with structural protein; whereas glutamic acid and alanine contents were sbustantially greater in soluble than structural protein (Tables 7,8). Additionally, some cultivar-specific variability in amino acid content of soluble protein(s) was evident (Table 8).

Inheritance of the Hull-less Mutant Condition

F_1 Testa Phenotypes

All F_1 seed from normal x hull-less mutant crosses ('Tricky

Table 8 Amino acid composition of Na-DOC-soluble protein in testa of normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of Cucurbita pepo.[a,e]

Amino Acid	Normal		Mutant	
	'Small Sugar'	'Jack O' Lantern	'Tricky Jack'	'293 A'
aspartic acid[b]	6.8	5.7	6.9	6.6
threonine[c]	2.8	2.2	2.6	2.5
serine[c]	4.1	3.4	4.3	3.9
glutamic acid[b]	22.8	36.4	28.8	20.5
proline[d]	3.8	2.5	5.5	4.3
glycine[c]	6.8	5.7	5.8	11.3
alanine[d]	10.3	16.2	13.1	11.8
½cystine[c]	0.1	3.3	0.1	0.1
valine[d]	4.4	0.9	4.1	4.1
methionine[d]	1.1	2.5	0.9	1.0
isoleucine[d]	3.0	4.3	3.1	2.9
leucine[d]	5.1	6.2	4.9	4.9
tyrosine[c]	2.7	1.4	2.4	11.4
phenylalanine[d]	2.4	1.5	2.2	2.3
histidine[b]	1.4	1.4	1.1	1.1
lysine[b]	2.9	2.8	3.0	3.0
arginine[b]	3.0	3.2	3.3	3.8

[a] Data are expressed as % composition of protein.

[b] Represents a polar, charged amino acid.

[c] Represents a polar, uncharged amino acid.

[d] Represents a non-polar amino acid.

[e] Analysis did not include hydroxyproline or tryptophan.

Jack' x 'Small Sugar'; '293 A' x 'Small Sugar'; and NH 61-22-19-12 x "Jack O'Lantern') exhibited the normally hulled phenotype. Crosses among different hull-less or partially hull-less strains resulted in seed progeny exhibiting the hull-less phenotype (e.g. 'Tricky Jack' x '293 A'; 'Triple Treat' x NH 10-2-28) (Fig. 5). However, the seed phenotypes of the progeny did not always resemble those of the parents. It was possible to obtain partial hull-less phenotypes from crosses of two completely hull-less parents.

F_2 Segregation for the Hull-less Characteristic

Four dry seed phenotypes were generally observed in F_2 and testcross seed populations which were segregating for the hull-less condition (Fig. 6). These phenotypes were arbitrarily classified as completely hulled, partially cream (thinly hulled over the entire seed surface), partially green (slightly hulled seed margin, but seed surface appears green because of the exposed chlorenchyma) and completely hull-less (seed appears green and margins are obscure). Some variability existed among classes and within crosses, and, thus, assignment of phenotypes was subjective. Table 9 shows the results obtained from the segregating and testcross populations. Testcross populations were smaller than planned due to either poor seed germination or woodchuck damage in the field. The NH 61-22-19-12 x 'Jack O'Lantern' testcross exhibited only 15% germination and was not planted in the field.

Grouping the various sub-divisions of the hull-less character

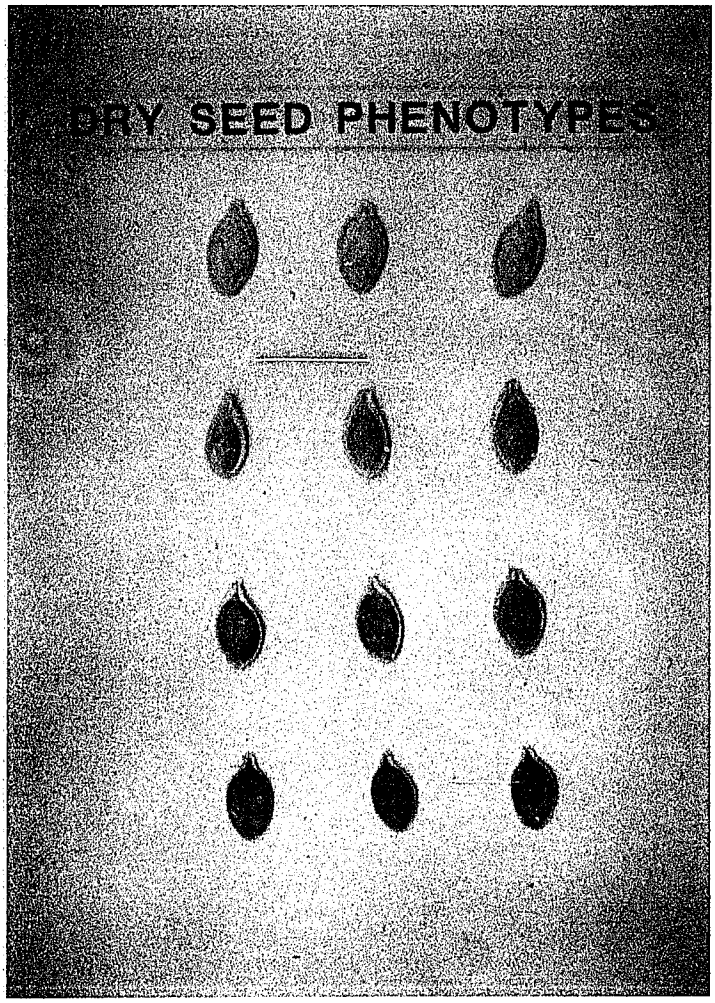


Figure 5. Dry seed phenotypes of F_2 and testcross populations of Cucurbita pepo.

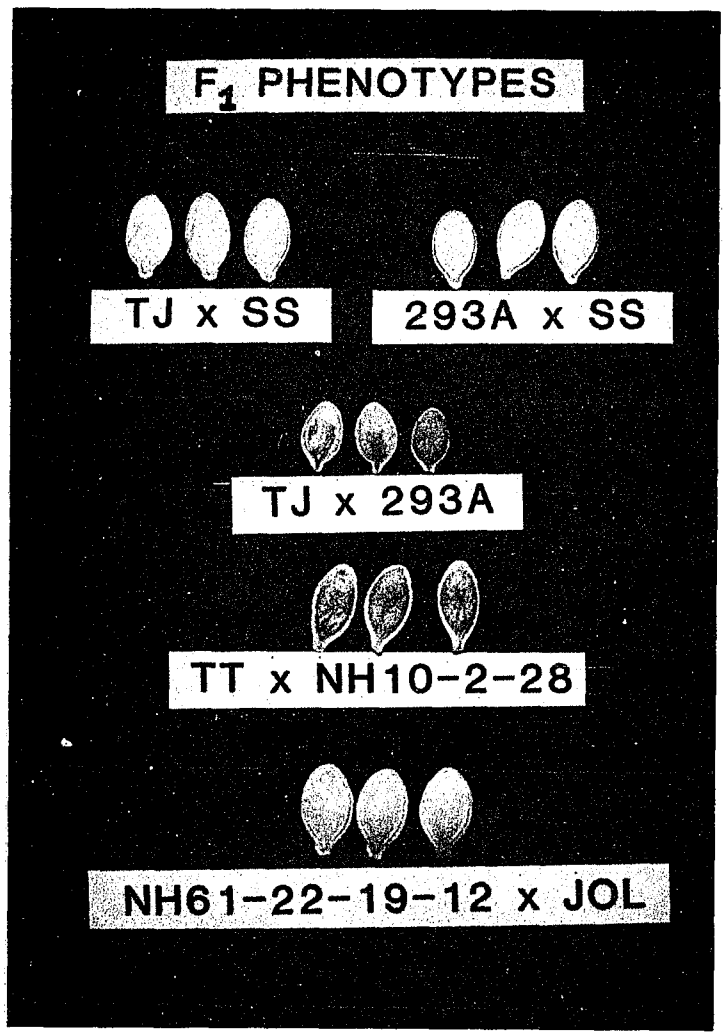


Figure 6. Dry seed phenotypes of F_1 populations of C. pepo.

Table 9. F_2 and testcross segregation for the hull-less seeded trait in Cucurbita pepo.

Crosses	Observed Phenotype: Normal	Observed Phenotype: Hull-less [a]	X^2	P
F_2's			X^2 (3:1)	
(293 A x SS)	101	27	1.04	50-30
(TJ x SS)	102	33	0.04	90-80
(61-22-19-12 x JOL)	93	33	0.17	70-50
Combined total	296	93	0.22	70-50
Testcrosses			X^2 (1:1)	
TJ x (TJ x SS)	26	24	0.08	80-70
293 A x (293 A x SS)	43	29	2.72	10- 5
Combined total	69	53	2.10	20-10

[a] Combined seed progeny of 3 classes of hull-less seeds (thin hull = partial cream; thin margin = partial green; completely hull-less = no hull).

together resulted in a 3 : 1 phenotypic ratio, i.e. 3 hulled to 1 hull-less (Table 9). This suggested a single recessive gene controlling partial hull-less expression. Testcross data confirmed this monofactorial inheritance hypothesis (X^2 = 2.10; P = 20-10), in that normal and hull-less phenotypes were recovered in roughly equal proportions.

The best chi square fit to account for all F_2 phenotypes was for three genes (X^2/ 48: 9:6:1), a single major gene plus two modifying genes which are tentatively being referred to as hull-less intensifiers. Table 10 shows the sub-classifications of the hull-less phenotypes, accompanied by putative genotypic explanations for each phenotype. Assuming that 'H' is a major gene governing hull formation, seed progeny which are homozygous or heterozygous for the major dominant gene,'H' will be completely hulled, regardless of the genetic constitution at the two modifying (hull-less intensifying) loci. Any hull-less or partially hull-less phenotype would be homozygous recessive at the major gene locus. However, the completely hull-less phenotype would only be obtained if both hull-less intensifiers were also present as homozygous recessive gene pairs. If both intensifiers were heterozygous, a thin-hulled (partial cream) phenotype would be observed. Finally, if either, but not both intensifying gene pairs was homozygous (recessive), a green seed with a slightly hulled margin would be obtained (partial green).

There is significant lack of fit of testcross data to the proposed single gene plus two modifier hypothesis (Table 10;

Table 10. Chi square analysis of F_2 and testcross populations of normal x hull-less mutant strains, based on a three gene model- one major gene and two modifying genes. Abbreviations: TJ = Tricky Jack; SS = Small Sugar; JOL = Jack O'Lantern.

Cross	Observed Phenotypes [a]				X^2	P
	Hulled	Partial Cream	Partial Green	Completely Hull-less		
F_2's					X^2(48: 9:6:1)	
(TJ x SS)⊗	101	13	12	2	1.65	70-50
(293 A x SS)⊗	102	19	12	2	0.09	100-95
(61-22-19-12 x JOL)⊗	93	17	13	3	0.68	90-80
Combined total	296	49	37	7	0.88	90-80
Testcross						
TJ x (TJ x SS)	26	12	8	4	8.63	5- 1
293 A x (293A x SS)	43	13	11	5	7.50	10- 5
Combined total	69	25	19	9	12.60	< 1

[a] Explanation of genotypes for corresponding phenotypes: let H - major gene conditioning hull formation and h = some degree of 'hull-lessness'; hi-1 and hi-2 are hull-less intensifiers (modifying genes). Hulled: H-hi-1 -; H-hi-1hi-1hi-2 ; H-hi-1 -hi-2 -; H-hi-1hi-1hi-2hi-2; Partial cream: hhhi-1 -hi-2 -; Partial green; hhhi-1hi-1hi-2 -; hhhi-1 -hi-2hi-2; Completely hull-less: hhhi-1hi-1hi-2hi-2.

$X^2 = 12.62$; $P < 1$). Also, hull-less seeded cultivars of C. pepo exhibit yearly and seasonal variability in degree of testa development (Figs. 7,8). If environment can affect shifts in phenotypic classes of the hull-less trait, then it may be possible to explain the inheritance on the basis of a two gene model.

Figure 7. Seasonal variability in expression of the hull-less phenotype in cvs. 293 A (fig. 7A) and Tricky Jack (fig. 7B).

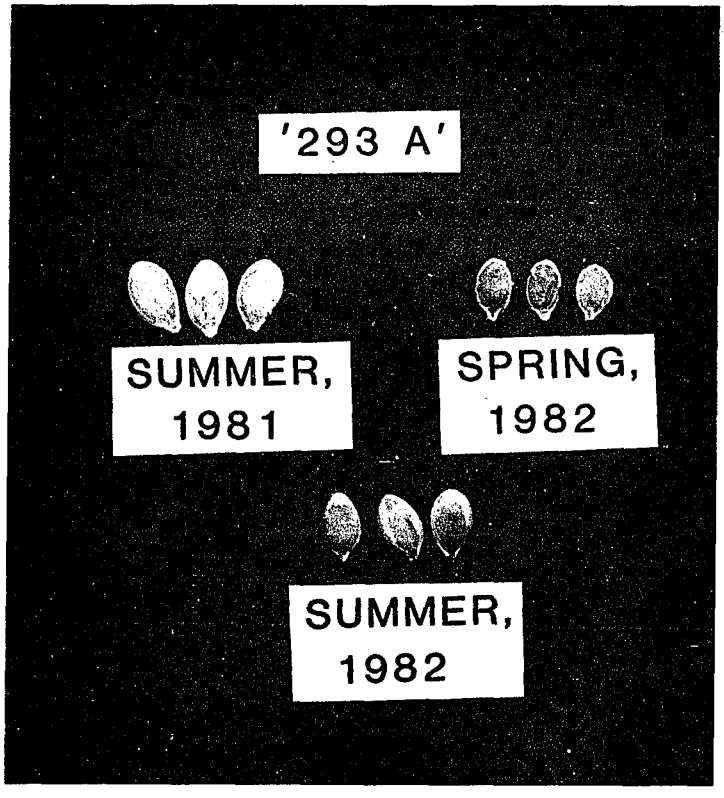


Figure 7A

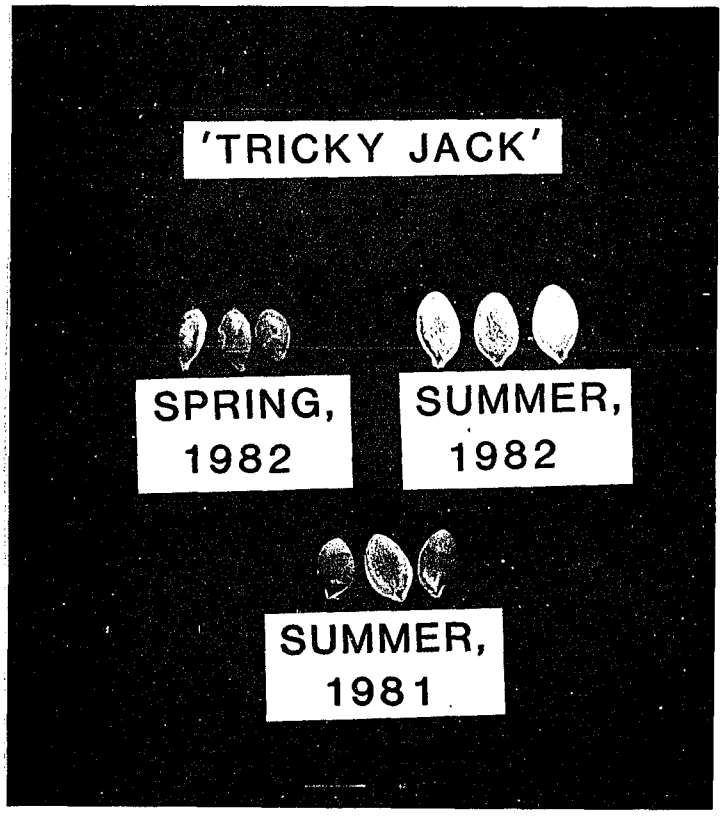


Figure 7B

DISCUSSION

Biochemical and Physiological Observations of Normal Versus Hull-less Metabolism

The testa of Cucurbita pepo clearly functions as a storage organ during seed development. This statement is substantiated by dry and fresh weight studies of the developing pumpkin testa and also by compositional analyses of normal and hull-less seeded strains of C. pepo (pumpkin). Testa dry weight is reduced by a remarkable 40-50% between 20 days post-anthesis and maturity in pumpkin seeds. Precursor materials then, as well as starch and lipids are apparently 'housed' in the developing testa at 20 days post-anthesis. It is doubtful that these precursors and reserves are incorporated into testae cell walls since wall polysaccharide and protein fractions have largely been synthesized by 20 days post-anthesis in normal and hull-less strains of C. pepo. Data suggest that lignin is the only wall fraction of the testa which is primarily manufactured between 20 days post-anthesis and maturity. Thus, testa reserves are most likely utilized by the developing embryo, particularly the abundant starch reserves (15-20% of testa dry weight at 20 days post-anthesis) in the chlorenchyma tissue of the testa. Additionally, if the developing embryo is receiving a good portion of its carbohydrate reserves from the testa, it is conceivable that protein precursors for embryogenesis could also be obtained from the testa.

Directly related to precursor utilization and the storage capacity of the developing testa is the substantial reduction or

'turn-over' of several structural testa cell wall fractions in normal and hull-less mutant strains between 20 days post-anthesis and maturity. Pectic polysaccharides are reduced by a remarkable 80-90% during this period while the diminution of hemicelluloses is substantial (50% for normal strains and 80% for hull-less strains). Since hull-less strains have considerably reduced wall synthesis in testae (Stuart, 1981; Stuart and Loy, in press), this difference between phenotypes is a predictable outcome, although such dramatic reductions are quite shocking. Good direct evidence is lacking to support this turn-over notion, but O'Brien (1975) working with protoplasts of tracheary elements stated that hydrolase activity could account for such degradation of cell wall components. Meier (1958) observed that endosperm cells of date and ivory nut store substantial quantities of hemicelluloses (primarily mannans). In any instance, the observed storage capacity of the developing testa of Cucurbita pepo is a unique and previously unreported phenomenon of seed development which should be viewed seriously in the context of embryogenesis and cell wall development.

It was anticipated that in terms of the genetics of the hull-less mutant an investigation of precursor pools might provide additional clues as to the mechanism(s) eliciting the hull-less phenotype (Stuart, 1981). No apparent quantitative differences in any of the studied precursors, reducing sugars, free amino acids, total phenolics or starch, were observed between developing normal and hull-less testae at 20 days post-anthesis. Starch is included here as an indirect precursor, whereby hydrolysis of the compound can

supply monosaccharide residues for either wall synthesis or embryogenesis. Related to the absence of quantitative differences between phenotypes at 20 days post-anthesis is the observation that no accumulation of any precursor was evident at maturity in either hull-less strain investigated. Many precursors may be shunted through alternative metabolic pathways not primarily involved in cell wall synthesis e.g. lipids biosynthesis. Precursor pools could also be utilized more extensively for embryogenesis in hull-less strains, since these strains have a reduced requirement for precursors for testa cell wall biogenesis.

The sole quantitative difference which could be putatively implicated in eliciting the hull-less phenotype in C. pepo lies in the amino acid composition of the structural protein species bound to or associated with the hemicellulose (xylan) fraction. At 20 days post-anthesis, protein bound to the hemicellulose 'B' fraction of the testa of hull-less strains contained a greatly reduced quantity of methionine, no detectable cysteine (both sulfur-containing amino acids), and increased amounts of valine compared with normal strains. Strong evidence supporting the role of cell wall glycoproteins in morphogenesis has been rapidly accruing (Preston, 1974; Lamport, 1970; 1973; Franz and Haas, 1980). "Without glycoproteins, there could be no extracellular matrix and without the matrix, there could be no morphogenesis as we know it" (Lamport, 1980). Because protein has been strongly implicated in cell wall biogenesis, it logically follows that wall structure could be relinquished if structural protein is reduced or is deficient in binding properties necessary for structural integrity or

polysaccharide incorporation into cell walls.

This inability of protein to bind to polysaccharides, or perhaps even to lignin as suggested by Harborne (1979) could reasonably and logically account for the deficiency of wall development in the hull-less phenotype of C. pepo. The primary composition of the structural protein (the amino acid sequence) could have been altered in hull-less strains, resulting in an alteration of tertiary structure.

Since both hull-less cultivars exhibited this deficiency of methionine and cysteine, and additionally, because they are genetically unrelated, it is extremely doubtful that the observation is purely coincidental. Moreover, a high degree of strain-specific variation exists in the amino acid composition of the structural protein associated with the xylan wall fraction of the testa. This observation coupled with the consistent phenotypic differences in amino acid composition of structural hemicellulose 'B' protein is potentially powerful evidence to support the notion of the hull-less genetic lesion primarily affecting some aspect(s) of protein synthesis. If screening many hull-less strains produces consistent similarities in the structural protein composition, this could ultimately verify several point mutations whose effects are manifest at the phenotypic level. A method for obtaining protein devoid of contaminating polysaccharide species must be devised to allow for separation of wall protein species in C. pepo, for fingerprint mapping of polypeptides, and ultimately, for amino acid sequencing of peptides which differ between normal and hull-less phenotypes. It

would also be desirable to examine the structural 'attachment' of such protein, i.e. to what polysaccharides does each species bind and to which individual monosaccharide residues.

There were no consistent differences in amino acid composition of sodium deoxycholate soluble protein between normal and hull-less strains. Additionally, no similarities in amino acid content between this soluble fraction and the structural fraction attached to the isolated hemicellulose 'B' were observed. In light of these observations, it follows that only a small proportion of the soluble protein could represent precursor material for wall protein synthesis, or alternatively, hemicellulose 'B' protein is largely synthesized by this stage of development. It is also conceivable that structural protein precursors are contained in buffer-soluble extracts of the testa (Loy, personal communication). Soluble protein which is not apparently utilized for wall synthesis could be degraded and eventually translocated and assimilated in the cotyledons since cucurbits generally store substantial amounts of globulins in mature cotyledons (Bewley, 1978).

In reviewing these new findings and in accord with previously presented anatomical and quantitative discussions of the hull-less phenotypes, the following progression of biochemical events is suggested to operate in hull-less strains of Cucurbita pepo. Primary wall development appears to proceed normally (in an anatomical sense) in hull-less strains until about 15 to 20 days post-anthesis. At this stage of development, hemicelluloses which are postulated for wall matrix formation to bind incoming protein (in addition to per-

haps cellulose) are present in equal quantities in both normal and hull-less phenotypes. Data suggest that the genetic lesion(s) of hull-less strains primarily affects the amino acid composition of protein associated with the hemicellulose 'B' wall fraction. This, in turn, could affect wall protein binding properties, which could ultimately disrupt secondary wall biogenesis in hull-less mutant strains of C. pepo. Thus, polysaccharide fractions which are not utilized in wall synthesis either as a source of precursor material or directly as polysaccharide matrix are degraded and the products translocated to other organs. The proposed degradation or turnover in hull-less strains must greatly exceed that of normal strains, since very large differences in testa dry weight exist between phenotypes at maturity. Because lignin, in addition to structural polysaccharides, has also been implicated in binding to protein, it follows that it too is markedly reduced in hull-less cell walls. This reduction should be proportionally greater than that of polysaccharide content since lignin is manufactured primarily during secondary wall development. Structural carbohydrates, however, particularly hemicelluloses and cellulose are synthesized throughout the course of primary and, to a much lesser extent, secondary wall biogenesis.

Inheritance of the Hull-less Phenotype

F_2 and testcross data indicate that a single major gene controls expression of the normal versus hull-less or partially hull-less testa characteristic. Normally hulled and hull-less phenotypes are recovered in roughly a 3:1 ratio. Additionally, it can be

suggested that one or two modifying genes which control the degree of expression of the completely hull-less phenotype interact with the major gene. Thus far in the inheritance study, data are in accord with the hypothesis presented by Grebenscikov (1954) and espoused by Whitaker (1962), favoring monofactorial inheritance of the testa condition and interaction of modifiers.

Problems arise in assigning genotypes to the variety of hull-less phenotypes since continuous phenotypic differences in the hull-less class are apparently not attributed solely to genetic differences between parents. Because of the observed seasonal and yearly variation in expression of the hull-less or incompletely hull-less phenoyptes in some breeding lines and cultivars, environmental interaction must, to some extent, be responsible for the range of hull-less phenotypes being observed.

The assigning of phenotypes was arbitrarily performed by grouping together the seed populations and subjectively deciding which types were similar. While some 'mis-assignment' may have contributed to the deviations from expected testcross ratios, this could not have accounted for all the variation.

Future investigations to observe the environmental effect on hull-less mutant gene expression will include larger testcross populations. Additionally, it will be desirable to observe growth of plants (and ultimately, testa formation) in a more controlled environment in greenhouses.

CONCLUSIONS

1. The physical parameters of testa development which were investigated, % moisture and dry and fresh weights, were similar. However, the substantial reduction in testa dry weight between 30 days post-anthesis and maturity was markedly greater in hull-less than normal strains of Cucurbita pepo.

2. Precursor pools for cell wall synthesis, namely free amino acids, reducing sugars, and total phenolics were not deficient in hull-less strains at 20 days post-anthesis. No accumulation of any precursor or reserve compound was observed for testae at maturity in hull-less strains.

3. Fractionation of testa components at 20 days post-anthesis revealed dramatic reductions in lignin and cellulose in the hull-less compared with the normal phenotype; whereas no marked differences were obtained for amounts of hemicelluloses, pectin, or hemicellulose-associated protein between normal and hull-less phenotypes.

4. A substantial turn-over or degradation of hemicelluloses and pectic polysaccharides occurred in testae of normal and hull-less strains of Cucurbita pepo. The turn-over in hull-less strains greatly exceeded that of normal strains.

5. Based on conclusions 1 and 4, the testa of C. pepo should be regarded as a storage organ during seed development, supplying nutrients for cell wall synthesis and embryogenesis.

6. Results of the inheritance investigation indicate that the hull-less developmental condition in testae of seeds of pumpkin (Cucurbita

pepo) is controlled by a single major gene, and the degree of hull-lessness may be affected by modifying genes (hull-less intensifiers). Variable expression of the hull-less phenotypes indicates some degree of environmental interaction with hull-less genotypes.

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APPENDIX

Appendix A. Analysis of soluble protein in sodium deoxycholate (Na-DOC) extracts of 20 day post-anthesis testae of seeds of Cucurbita pepo.

400 µl aliquots of Na-DOC extracts were pipetted into 16 x 200 mm test tubes and 100 ml of 1% phosphoric acid was added to each tube. Following almost immediate precipitation of 'protein', mixtures were centrifuged at 480 x g in a Sorvall bench top centrifuge and placed in an oven at 80 C for 48 h. Dried material was resuspended in 400 µl of 80% ethanol, the mixtures vortexed well and 100 ul aliquots used for Bradford analysis of soluble protein. Bovine serum albumin was used to construct the standard curve.

Appendix B. Extraction of testa precursor pools using the MCW extraction procedure (Haissig and Dickson, 1979).

Testa tissue (dried and weighed samples) was extracted repeatedly with sonication in methanol:chloroform:water (MCW), 12:5:3, vol/vol for a total of three times. Following each extraction, the mixtures were centrifuged at 480 x g for 15 min, and the supernatant fluid was pipetted off and saved. After the final extraction, supernatants were combined and 3 ml of double distilled, deionized water was added for each 5 ml of solution. The mixtures were vortexed for 15 sec, and centrifuged at 480 x g. The chloroform pigment/lipid phase was separated from the polar methanol:water phase and the latter was frozen at -10 C for subsequent amino acid, sugar and phenol analyses as described in MATERIALS AND METHODS.

Appendix C. % nitrogen for amino acid constituents and total protein of hemicellulose 'B'-bound protein of testae of normal and hull-less strains of C. pepo.

	Normal		Mutant	
amino acid	'SS'	'JOL'	'TJ'	'293 A'
asp	.01421	.01305	.01421	.01158
thr	.00271	.00306	.00435	.00353
ser	.00440	.00453	.00627	.00547
glu	.01181	.01200	.01219	.01000
pro	.00304	.00487	.00487	.00438
gly	.00643	.00793	.00550	.00593
ala	.00708	.00818	.01164	.00944
cys	.00532	.00590	-	-
val	.00084	.00084	.00933	.00730
met	.00282	.00338	.00103	.00075
ile	.00545	.00684	.00566	.00432
leu	.01453	.00737	.00940	.00780
tyr	.00325	.00333	.00410	.00255
phe	.00221	.00263	.00399	.00305
his	.00867	.01707	.00596	.00705
lys	.01074	.01170	.01074	.00940
arg	.03025	.01030	.01095	.04023
Total (x 100)	13.38	12.30	12.87	13.28

Appendix D. % nitrogen for amino acid constituents and total protein of Na-DOC osluble protein of testae of normal and hull-less strains of C. pepo.

	Normal		Mutant	
amino acid	'SS'	'JOL'	'TJ'	'293 A'
asp	.00716	.00600	.00726	.00695
thr	.00295	.00259	.00306	.00294
ser	.00547	.00453	.00573	.00520
glu	.02172	.03475	.02743	.01952
pro	.00462	.00304	.00669	.00523
gly	.00486	.00407	.00414	.00807
ala	.01620	.02548	.02061	.02328
cys	.00012	.00382	.00012	.00012
val	.00526	.06108	.04906	.04906
met	.00103	.00245	.00085	.00094
ile	.00321	.00459	.00331	.00310
leu	.00545	.00662	.00524	.00524
tyr	.00209	.00108	.00186	.00882
phe	.00204	.00127	.00187	.00195
his	.00380	.00380	.00298	.00298
lys	.00556	.00537	.00575	.00575
arg	.00966	.01030	.01062	.01223
Total (x 100)	10.12	11.81	15.66	13.67

Appendix E. Micrograms of protein in hemicellulose 'B' wall fractions of normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of C. pepo.

	Normal		Mutant	
	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
µg protein (Hc_B-associated)	105.88	180.88	240.38	94.75

Appendix F. Amino acid composition (nmols/mg sample) of Na-DOC soluble and hemicellulose 'B'-bound protein hydrolyzates of testae of 20 day post-anthesis normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of C. pepo.

	Normal				Mutant			
	'SS'		'JOL'		'TJ'		'293 A'	
amino acid	DOC	HcB	DOC	HcB	DOC	HcB	DOC	HcB
asp	28.72	18.91	40.05	19.48	46.87	29.86	50.09	12.03
thr	11.69	3.17	15.53	4.07	17.87	8.19	19.19	3.25
ser	17.15	4.62	25.05	5.36	28.98	10.48	29.87	4.45
glu	95.89	17.36	253.87	19.82	194.12	28.27	155.44	11.38
pro	16.09	4.08	17.30	6.27	36.85	8.76	32.66	3.88
gly	28.52	12.65	40.11	17.62	38.80	17.12	85.87	8.98
ala	43.60	6.30	112.95	8.14	88.46	16.29	111.87	6.48
cys	0.57	6.46	23.55	8.09	0.98	-[a]	1.00	-[a]
val	18.67	0.92	6.28	1.08	28.01	17.34	31.55	6.57
met	4.74	4.27	17.51	5.70	5.92	2.52	7.77	0.92
ile	12.63	7.19	29.99	10.04	20.60	11.73	22.17	4.47
leu	21.34	19.09	43.49	10.91	32.91	19.51	37.13	7.92
tyr	11.21	5.82	10.01	6.82	16.52	11.85	86.12	3.60
phe	10.28	3.64	10.35	4.85	15.28	10.30	17.40	3.93
his	5.86	4.48	9.91	4.36	7.30	4.86	8.55	2.82
lys	12.15	7.88	19.44	9.63	20.20	12.35	23.26	5.28
arg	12.63	13.22	22.56	5.18	22.22	7.56	28.52	13.53

[a] None was detected for the injected sample.

Appendix G. Free amino-containing compounds soluble in phosphate buffer, sodium deoxycholate (Na-DOC) and sodium chloride extracts of 20 day post-anthesis testae of normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of C. pepo.

	Normal		Mutant	
Extract	'Small Sugar'	'Jack O' Lantern'	'Tricky Jack'	'293 A'
PO_4 buffer (mg/organ)	2.73 ± 0.04 [a]	2.48 ± 0.25	1.86 ± 0.49	2.90 ± 0.14
Na-DOC (mg/organ)	0.08 ± 0.02	0.23 ± 0.01	0.17 ± 0.04	0.10 ± 0.05
NaCl (mg/organ)	1.63 ± 0.35	1.19 ± 0.14	1.58 ± 0.37	1.38 ± 0.06

[a] Values ± SE represent the mean of 2 samples/strain (3 replicates within each sample).

Appendix H. Amino acid composition of hemicellulose 'B'-bound protein of testae of normal ('Small Sugar' and 'Jack O'Lantern') and hull-less mutant ('Tricky Jack' and '293 A') strains of Cucurbita pepo.

	Normal				Mutant			
	'SS'		'JOL'		'TJ'		'293A'	
amino acid	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
asp	13.5	13.5	12.4	11.4	13.5	12.6	11.0	10.6
thr	2.3	2.1	2.6	2.6	3.7	3.3	3.0	2.9
ser	3.3	3.6	3.4	3.5	4.7	4.2	4.1	4.4
glu	12.4	12.4	12.6	13.0	12.8	13.3	10.5	10.3
pro	2.9	3.6	4.0	5.7	4.0	6.6	3.6	4.1
gly	9.0	8.9	11.1	10.5	7.7	7.5	8.3	8.7
ala	4.5	4.6	5.2	5.5	7.6	6.7	6.0	5.8
½ cys	4.6	- [a]	5.1	- [a]	- [a]	- [a]	- [a]	- [a]
val	0.7	4.7	0.7	5.2	7.8	7.2	6.1	6.0
met	3.0	1.0	3.6	0.9	1.1	1.1	0.8	1.1
ile	5.1	2.9	6.4	3.9	5.3	4.7	4.1	3.9
leu	13.6	4.9	6.9	7.0	8.8	7.8	7.3	6.6
tyr	4.2	7.3	4.3	3.8	5.3	3.8	4.1	5.6
phe	2.6	3.8	3.1	3.3	4.7	3.8	3.6	4.6
his	3.2	3.1	6.3	2.7	2.2	1.9	2.6	2.3
lys	5.6	5.2	6.1	5.8	5.6	5.4	4.9	4.9
arg	9.4	2.5	3.2	7.9	3.4	2.8	12.5	2.4

[a] None was detected for the injected sample.

[b] Abbreviations: 'SS'- Small Sugar; 'JOL'- Jack O'Lantern; 'TJ'- Tricky Jack; '293 A'- 293 A.